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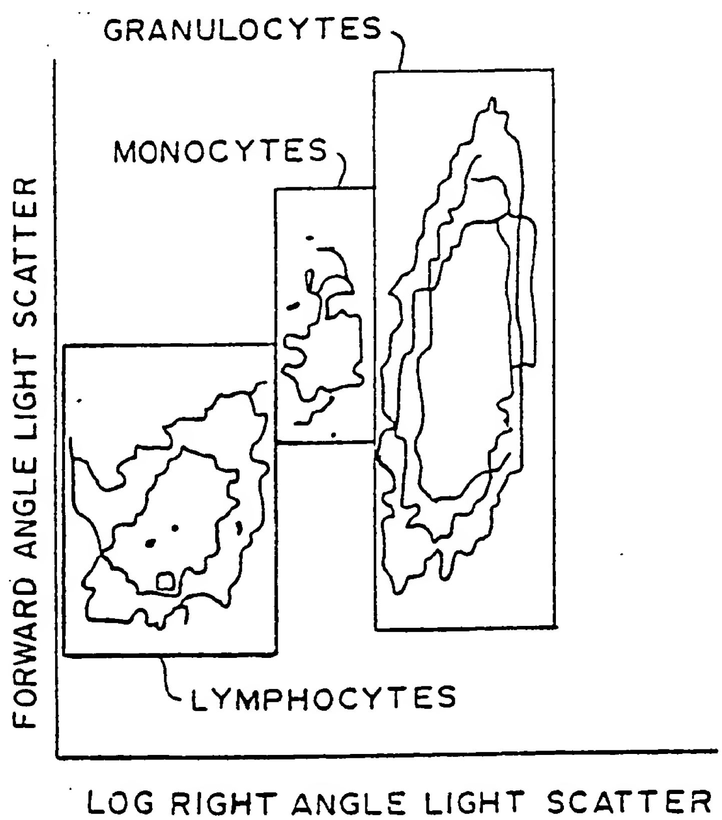
(54) Title: A REAGENT SYSTEM AND METHOD FOR IDENTIFICATION, ENUMERATION AND EXAMINATION OF CLASSES AND SUBCLASSES OF BLOOD LEUKOCYTES

(57) Abstract

Specified classes and subclasses of leukocyte blood cells are identified by immunohematology procedures, based on utilization of antigenic determinants on the cell surface, their reactivity with antibodies which fluoresce under known circumstances, and identified by utilization of principles of flow cytometry or morphology. This invention particularly concerns improvements in the lysing and fixing method used prior to detection and identifying of the cells. In this method, a whole blood sample first is incubated with a reagent including antibodies to the cell subclass to be identified, the antibodies having been directly or indirectly made fluorescently responsive to a particular light (e.g. argon ion laser). The red blood cells then are lysed with a reagent containing saponin. Next follows a leukocyte fixing treatment, preferably using a cross-linking dialdehyde composition, such as glyoxal or glutaraldehyde. Details are given as to time, temperature, concentration, and the use of additives and stains, as well as the use of particular monoclonal antibodies in the preferred procedure.



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A REAGENT SYSTEM AND METHOD FOR IDENTIFICATION, ENUMERATION AND EXAMINATION OF CLASSES AND SUBCLASSES OF BLOOD LEUKOCYTES

This invention relates to immunohematology procedures, and more particularly to a reagent system and a method for the identification of classes as well as subclasses within a class and enumeration of cells within those subclasses of blood leukocytes from a whole blood sample which has been incubated with a fluorescent responsive antibody to a select antigenic determinant on the surface of specified subclasses of blood leukocytes.

It is known that the lymphocyte population of blood leukocytes is subdivided into a number of subclasses which play distinct roles in the immune response. In disease states the relative number of lymphocytes found in various subclasses is likely to change. Hence, the enumeration and identification of the cells in the various subclasses will provide useful information in the study and treatment of disease as described by James R. Downing et al in Laboratory Management, May 1984, pages 29-37.

It is known that several particular subclasses of functionally distinct lymphocytes and other blood leukocytes can be identified on the basis of antigenic determinants on the cell.

Monoclonal antibody techniques have been utilized to produce large quantities of highly purified antibody to various lymphocyte and other leukocyte subclasses. Utilizing such antibodies, it has proved feasible to assay the lymphocytes of an individual to determine the relative number of cells in various subclasses. Further, utilizing direct or indirect techniques, the antibodies can be labeled fluorescently, thereby rendering the samples under consideration amenable to flow cytometric analysis and morphology. More recently, additional monoclonal antibodies have been developed which include several that react with monocytes and granulocytes.

Hansen et al describe in U. S. Pat. 4,284,412, 1981, and in Immunology, Vol. 77, No. 8, pp 4914-4917 (1980) a method and apparatus for automated identification and enumeration of specified lymphocyte subclasses. An anticoagulated whole blood sample or buffy coat sample is incubated with an antibody to a specific lymphocyte subclass of interest. The binding of this antibody is detectable if either it has been coupled with a fluorescent chemical moiety (the direct

technique), or if it in turn is specifically bound by another macromolecule to which has been coupled a fluorescent dye moiety (the indirect technique). These fluorescent moieties possess the characteristic of emitting fluorescent light upon illumination with incident laser light. The sample then is lysed using ammonium chloride as the lysing agent. A diluted sample then is subjected to flow cytofluorometric analysis. Four clusters of cells are distinguished. However, only three clusters are found to be due to leukocytes. These clusters were identified as (1) lymphocytes, (2) monocytes and (3) granulocytes. The fourth cluster is identified as aggregates or multiples of platelets and red blood cell debris due to incomplete lysing of the red blood cells.

The lysing techniques described in these references now have been improved by the present invention so as to maintain better the morphology of the immunologically labeled specific leukocytes, improve their stability on storage, and render them more suitable for cytofluorescent analysis, or for other operations such as microscopic examination of stained cells on a slide.

Employed herein are the trademarks COULTER, COULTER CLONE, ISOTON, E.A.SY.1, MDADS and EPICS which are owned by Coulter Corporation, Coulter Electronics, Inc., or Coulter Electronics, Ltd.

By way of example, illustrative embodiments of the invention now will be described with reference to the accompanying drawings by which:

Figs. 1A and 1B show the cell-membrane-antigen histograms produced by flow cytometric analysis. The abscissa represents a value directly proportional to the log of the green fluorescence intensity. The ordinate represents the number of cells counted in each channel on a relative scale.

Fig. 1A shows a sample with T4-FITC, as in Example 1.

Fig. 1B shows a control with no fluorescent antibody added.

Fig. 2 is a contour map in which the abscissa represents the log of the right angle light scatter intensity, and the ordinate represents the forward angle light scatter intensity. The rectangles designate the specific areas of this two dimentional distribution

which contain the specific leukocyte cell classes: lymphocytes, monocytes, granulocytes. This contour map is determined from the two dimensional data by a flow cytometer. Each contour line designates the locations on the map where there is essentially the same number of cells, irrespective of light scatter intensity.

This invention relates to an improved reagent system and method for identifying classes and enumerating the cells in selected subclasses within those classes of leukocytes in whole blood, based on utilization of antigenic determinants on the cell surface and their reactivity with labeled antibodies. This invention employs somewhat the general overall procedure described by Hansen et al, but with improvements especially over the conventional lysing and cell fixing procedure.

According to the present invention, the reagent system comprises aqueous solutions of (A) a lysing reagent containing saponin, and (B) a fixing reagent containing a cross-linking agent. The improved reagent system lyses the blood erythrocytes, while maintaining the physical and morphological properties of the blood leukocyte classes, as well as labeling specific leukocyte subclasses. The samples can be analyzed either by the principles of flow cytometry, or by microscopic morphology.

Using saponin as the lysing agent, the method permits fixation with a limited amount of a cross-linking fixative. This is accomplished by lysing at room temperature, or preferably at an elevated temperature, such as 42°C, which selectively destabilizes the erythrocyte membrane and accelerates the lysing reaction. The use of a hypotonic buffer, consisting primarily of potassium salts, also favors lysis.

Glyoxal is preferred over glutaraldehyde as the cross-linking agent, even though the latter is a stronger fixative; because glyoxal, after reacting with a leukocyte, imparts minimal, if any, background fluorescence; whereas glutaraldehyde produces a significant amount of fluorescence. Dialdehydes are preferred over monoaldehydes, such as formaldehyde, which have only a weak cross-linking action for this purpose.

The addition of dimethyl sulfoxide or urea to the fixative improves the reaction, and tends to decrease the amount of fixative required to retard the action of the saponin on the leukocytes. In addition, a temperature drop to approximately 4°C (as by use of an ice bath) slows down the reaction. The cells are weakly fixed and can be sufficiently spread on a slide for cytological preparation.

In copending U. S. Application, S.N. 615,966, filed the same day as the present application, a somewhat analogous lysing method is described, but such method does not include immunological techniques for utilizing antigenic determinants on the cell surface and their reactivity with antibodies that label cells which fluoresce under known circumstances.

This invention concerns a method for identifying classes and enumerating cells in subclasses of leukocytes in whole blood, based on the utilization of antigenic determinants on the cell surface and their reactivity with labeled antibodies, followed by utilization of the principles of flow cytometry or microscopic morphology to identify the cells which have been labeled.

Employed herein are the terms "lysis", or forms thereof, and "morphology". Lysis of the erythrocytes means to render the erythrocytes such that they no longer are detectable by physical techniques, or the erythrocyte residue is such that the signals they generate are sufficiently decreased that they will not interfere with those produced by the blood leukocytes. Morphology means the process of microscopic examination of cells and the determination of their classes and subclasses by either human or artificial intelligence techniques.

According to this invention, a whole blood sample is incubated with diluted monoclonal antibody to label certain subclasses of leukocytes. The erythrocytes then are removed by lysis, and the leukocytes are stabilized by use of a cross-linking fixative, prior to the class and subclass identification. It is preferable to lyse the red blood cells before the steps of identifying the class and subclasses of leukocytes, to avoid the danger that coincident passage of two or more erythrocytes or fragments thereof through counting

transducer could be mistaken for white blood cells. A preferred procedure is to lyse the red blood cells prior to the identification of the leukocytes by addition of a lysing reagent to the suspension of cells so as to cause the red blood cells to rupture and release their hemoglobin content into the solution.

The problem is to lyse the red blood cells without damage to the antigen-antibody complexes on the leukocytes, while preserving their cell morphology and producing a stable cellular suspension.

The whole blood sample must be treated in a way that lyses the red blood cells, and at the same time the leukocyte blood cells are maintained in a condition which allows measurements to differentiate them and their subclasses. The lysing reagent needs to act quickly, preferably in less than one minute. The cytogram produced should have the cluster of aggregates or multiplets of platelets, and red cell debris separate from the leukocyte clusters.

In a preferred embodiment of the invention, the whole blood sample is incubated with a fluorochromed antibody to label a specific subclass of leukocytes. The erythrocytes are selectively lysed with a lysing reagent containing saponin, and the directly labeled leukocytes are then fixed, using a fixing reagent containing a dialdehyde. The leukocyte cell suspension then is analyzed by the combination of forward angle and right angle light scattering to yield data representing at least three classes. The positively immunofluorescent leukocyte populations are enumerated with a COULTER® EPICS® flow cytometer. The fixative is a cross-linking or bifunctional fixative, preferably a dialdehyde.

Successful results have been obtained with the following COULTER CLONE® antibodies: T4-FITC, T8-FITC, T11, T11-FITC, B1-FITC and Mo2-FITC. Other antibodies useful for this purpose would include OKT1.PAN, OKT4.IND, and also OKM1.M/G, which reacts with monocytes and granulocytes.

As used herein "fluorescent responsive antibody" refers to antibodies which themselves fluoresce, or antibodies which are labeled to fluoresce under specified stimulation.

A technique has been developed to lyse the blood erythrocytes, while maintaining the physical properties and morphology of the blood leukocytes, and immunologically labeling specific leukocyte subclasses. This technique is unique in that it does not involve centrifugation; yet, it does preserve the cells sufficiently so that they can be stored at 4°C for a day and still be usable for flow analysis. Using this technique, it is possible, for example, to divide T lymphocytes into (1) helper lymphocytes, using COULTER CLONE T4-FITC monoclonal antibody, and (2) suppressor lymphocytes, using COULTER CLONE T8-FITC monoclonal antibody.

The cells prepared by the techniques of this invention also are suitable for other cytological preparations, for instance by centrifugal cytology.

The technique of this invention minimizes the concentration of the saponin lysing reagent required, and consequently permits fixation with a limited amount of fixative. Lysing is accomplished at room temperature, or preferably at an elevated temperature such as 42°C, which selectively destabilizes the erythrocyte membrane and accelerates the lysing reaction. The use of a hypotonic buffer, consisting primarily of potassium salts, also favors lysis.

It has been found that the amount of saponin employed under comparable conditions of lysis can be decreased as the temperature is increased. Thus, the residual amount of saponin in preparations that are not centrifuged can be minimized by increasing the temperature of the lysing reagent.

Bifunctional or cross-linking fixing agents such as glyoxal, glutaraldehyde, carbodiimide, succinaldehyde, Mirsky's reagent, and the like, are suitable for use in the fixing reagent. Mirsky's reagent consists primarily of an impure preparation of glyoxal derived from the chemical treatment of saccharides. Glyoxal is especially preferred in the fixing reagent, because it does not fluoresce. This is an advantage when determining the fluorescence of the antibody binding cells later on in the procedure.

The invention is not limited to fluorescence measurements, but absorbance measurement can be performed such as is described in

"Defined Immunofluorescence and Related Cytochemical Methods" by D. Y. Mason, Z. Abdulaziz and B. Falini, Annuals of the New York Academy of Science, Vol. 420, pages 127-138 (1983).

The technique of this invention can be employed with the additional physical measurements of DC and AC impedence. The measurements can be utilized in conjunction with the aforementioned light scattering measurements, or in place of them.

The fixed blood cells are stable when stored cold at about 2 to 4°C prior to the light scattering procedure. A laboratory study established that samples were stable for 24 hours post lyse. For example, using COULTER CLONE antibody T8-FITC, the percent positive cells was 20.3 ± 1.0 immediately after sample preparation, 20.3 ± 0.6 after 20 hours post lyse, and 20.0 ± 0.3 at 24 hours post lyse.

Centrifugation and removal of the supernatant fluid between processing steps is beneficial in that it significantly decreases cell debris, and removes excess loosely bound antibody. However, centrifugation has the disadvantage that it virtually precludes obtaining an absolute count of the cells present, and under some conditions can result in selective cell loss. Although centrifugation can be part of a semiautomated system, it introduces considerable complexity into a completely automated system.

One of the major constraints in the development of the present reagent system is that sample preparation for a flow cytometer, such as an EPICS system, unlike that for a standard hematology analyzer, such as the COULTER® Model S series instrument, is asynchronous. The time between the final sample preparation and measurement can range from almost immediately to the next day.

One other problem is the choice of a suitable control. The exquisite specificity of monoclonal antibodies in some cases for example, when fluoresceinated mouse immunoglobulin G (mouse IgG-FITC) is used as a control, leads to an over-estimation of background fluorescence. Either a preincubation and/or a simultaneous incubation with unlabeled antibody blocks the nonspecific binding sites, and this in many cases can eliminate the need for a control.

Samples containing antibody prepared according to this invention are capable of being prepared employing Romanowsky stain. It is also possible in the case of fluorescent studies to stain the cell with two stains, such as dichlorofluorescin and 4,6-diamino-2-phenylindole (DAPI). Other conventional stains include Mithromycin and Acridine Orange.

The examples which follow illustrate certain of the methods and procedures followed in the invention.

Example 1

Cell Preparation: The lyse and fixative reagents are prepared at room temperature, approximately 24°C.

1. The 100 ul of phosphate buffered saline (PBS) is added into a 16 x 100 tube followed by 100 ul of whole blood. The tube contents then are gently mixed by swirling.

2. The 5 micrograms of COULTER CLONE monoclonal T11 antibody is added into the same tube and mixed gently. The tube then is kept at room temperature approximately 24°C, for 20 minutes, with occasional shaking.

3. The sample, since this was an indirect fluorescence technique, was washed twice by centrifugation with 4 ml PBS. The sample then was treated with 85 micrograms of fluoresceinated goat antimouse antibody and incubated for 20 minutes, and washed as before with 4 ml of PBS.

4. The cells were suspended in 1 ml of a solution consisting of 3 g NaCl and 1 g $NaHCO_3$ and water to one liter.

5. The 100 ul of lyse reagent consisting of: 24 g saponin, 4.0 g NaCl, 1 g sorbic acid and water to one liter then is added to the tube containing blood, including the indirectly labeled T lymphocytes and continuously agitated for eight seconds.

6. At the end of the 8 second lysis, 1000 ul of the fixative reagent is added to the lysed sample. This fixative reagent consisted of: 6.0 g of NaCl, 22 g of glutaraldehyde, brought up to one liter volume with water.

7. The sample which is indirectly labeled with monoclonal antibody, lysed and then fixed must be analyzed with the flow

cytometer within 15 minutes, because of the development of glutaraldehyde induced autofluorescence. Filtration of the sample, preferable through a 37 micron mesh, is desirable.

Sample Analysis: The samples are analyzed with the COULTER EPICS V single laser, flow cytometer system. The system configuration is set up as herein explained. The laser emits 500 mw of 488 nm radiation. the preferred filter configuration is a 515 interference acting as a blocking filter for green fluorescence, 488 nm dichroic mirror and an ND1 filter for orthogonal light scatter, and a ND1 filter for forward angle light scatter. The data are analyzed with a computer system, such as in the COULTER MDADS™ or E.A.SY.1®. The three parameters measured are log fluorescence, low angle light scatter and log right angle light scatter. This analysis procedure also is utilized for each of the following Examples.

Example 2

Cell Preparation: The lyse and fixative reagents are prepared at 37°C. The reagents can be kept capped in a water bath throughout the procedure.

1. A 100 ul portion of labeling diluent, consisting of 1.0 g of NaN_3, 1.36 g KH_2PO_4, 1.31 g of K_2HPO_4 and 3.73 g of KCl, brought up to one liter volume with water, is added into a 16 x 100 tube, followed by 100 ul of whole blood. The tube contents are then gently mixed by swirling.

2. Ten micrograms of a non-specific, unlabeled mouse antibody is added to block any non-specific binding of the monoclonal antibody.

3. One microgram of COULTER CLONE monoclonal antibody T4-FITC is added into the same tube and mixed gently. The tube is then placed in a water bath maintained at 37°C for five minutes, with occasional shaking.

4. The 100 ul of a lyse reagent consisting of 4 g saponin, 1.75 g of NaCl, 1.36 g of KH_2PO_4, 1.31 g of K_2HPO_4 and 2.24 g of KCl, brought up to one liter volume with water, is then added to the tube containing blood and antibody, and continuously is agitated in the water bath for one minute.

5. At the end of the one minute lysis, 500 ul of a fixative reagent is added to the lysed sample, mixed gently, and kept in the water bath at 37°C for an additional five minutes, mixing occasionally. This fixative reagent consists of 11.7 g of NaCl, 0.43 g of calcium gluconate, 21 g of glyoxal, 220 g of dimethyl sulfoxide and 25 g of Carbowax 1450 brought up to one liter volume with water.

At this point in time the whole blood has been labeled with monoclonal antibody, lysed and then fixed. It is now ready for analysis with the EPICS flow cytometer. Filtration of the sample, preferably through a 37 micron mesh, is desirable. The samples are stabilized at approximately 2°C on ice, if need be, for at least 5 minutes prior to sample analysis. Analysis is as stated at the end of Example 1.

In accordance with the above procedure, but substituting for the COULTER CLONE monoclonal T4-FITC of Example 1, any one of the following COULTER CLONE monoclonal antibodies: T8-FITC; T11-FITC; B1-FITC; Mo2-FITC; similar results are obtained. In each instance the leukocyte subclass which is labeled is the leukocyte which is specific for the antibody employed.

Example 3

Cell Preparation: The lyse and fixative reagents are prepared at 24°C. The reagents can be kept capped in a water bath throughout the procedure.

1. The 100 ul of a labeling diluent consisting of 1.0 g of NaN_3, in 1 liter ISOTON® Plus diluent is added into a 16 x 100 tube followed by 100 ul of whole blood. The tube contents are then gently mixed by swirling.

2. COULTER CLONE monoclonal antibody T4-FITC is added in the amount of one microgram into the same tube, mixed gently, and then placed in a test tube rack at room temperature (24°C) for five minutes, shaking occasionally.

3. The 1000 ul of lyse reagent, consisting of 0.5 g of saponin, 3.72 g of KCl, 1.36 g of KH_2PO_4, and 1.31 g of K_2HPO_4, brought up to one liter volume with water, is then added to the tube

containing blood and antibody, vortexed gently for 30 seconds and placed in a rack at room temperature (24°C) for five minutes, mixing gently each minute.

4. At the end of the five minute lysis, 1000 ul of the fixative reagent consisting of 12.6 g of NaCl, 220 g of dimethyl sulfoxide, 200 ml of Mirsky's reagent and 600 ml of ISOTON Plus diluent, is added to the lysed sample, mixed gently, and kept at room temperature (approximately 24°C) for an additional five minutes, mixing occasionally. Mirsky's reagent is commercially available from Mirsky's National Diagnostics, Somerville, New Jersey.

At this point in time the whole blood has been labeled with monoclonal antibody, lysed and then fixed. It is ready for analysis with the EPICS flow cytometer. Filtration of the sample, preferably through a 37 micron mesh, is desirable.

The samples are stabilized on ice, if need be, for at least 5 minutes prior to sample analysis, and analyzed as stated at the end of Example 1.

Example 4

Cell Preparation: The lyse and fixative reagents are prepared at 42°C. The reagents can be kept capped in a water bath throughout the procedure.

1. The 100 ul of a labeling diluent, consisting of 1.0 g of NaN_3 in 1 liter of ISOTON Plus diluent, is added into a 16 x 100 tube followed by 100 ul of whole blood. The tube contents are then gently mixed by swirling.

2. Four micrograms of COULTER CLONE monoclonal antibody T4-FITC are added into the same tube, mixed gently, and then placed in the water bath maintained at 42°C for five minutes, shaking occasionally.

3. The lyse reagent, consisting of 24 g of saponin, 4.0 g of NaCl, 1.0 g sorbic acid and water to make 1 liter is prepared. Ten milliliters of this lyse reagent is then diluted with 1 liter of lyse diluent consisting of 1.31 g K_2HPO_4 and 1.36 g KH_2PO_4 in distilled water. 1000 ul of the mixture is added to the tube containing blood and antibody, and the tube is continuously agitated in the water bath for one minute.

4. At the end of a 30 second lysis, 1000 ul of the fixative reagent consisting of 12.6 g of NaCl, 200 ml of Mirsky's reagent, 220 g of dimethyl sulfoxide, and 600 ml of ISOTON Plus diluent brought up to 1 liter volume with water, is added to the lysed sample, mixed gently, and kept in the water bath at 42°C for an additional five minutes, mixing occasionally.

At this point in time the whole blood has been labeled with monoclonal antibody, lysed and then fixed. It is ready for analysis with the EPICS flow cytometer. Filtration of the sample, preferably through a 37 micron mesh, is desirable.

The samples are stabilized on ice, if need be, for at least 5 minutes prior to sample analysis and analyzed as stated at the end of Example 1.

Example 5

Cell Preparation: The lyse and fixative reagents are prepared at 37°C. The reagents can be kept capped in a water bath throughout the procedure.

1. A 100 ul portion of labeling diluent, consisting of 1.0 g of NaN_3, 1.36 g KH_2PO_4, 1.31 g of K_2HPO_4 and 3.73 g of KCl, brought up to one liter volume with water, is added into a 16 x 100 tube, followed by 100 ul of whole blood. The tube contents are then gently mixed by swirling.

2. Ten micrograms of a non-specific, unlabeled mouse antibody is added to block any non-specific binding of the monoclonal antibody.

3. One microgram of COULTER CLONE monoclonal antibody T8-FITC is added into the same tube and mixed gently. The tube is then placed in a water bath maintained at 37°C for five minutes, with occasional shaking.

4. The 100 ul of a lyse reagent consisting of 4 g saponin, 1.75 g of NaCl, 1.36 g of KH_2PO_4, 1.31 g of K_2HPO_4 and 2.24 g of KCl, brought up to one liter volume with water, is then added to the tube containing blood and antibody, and continuously is agitated in the water bath for one minute.

5. At the end of the one minute lysis, 500 ul of a fixative reagent is added to the lysed sample, mixed gently, and kept in the

water bath at 37°C for an additional five minutes, mixing occasionally. This fixative reagent consists of 11.7 g of NaCl, 0.43 g of calcium gluconate, 21 g of glyoxal, 220 g of dimethyl sulfoxide and 25 g of Carbowax 1450 brought up to one liter volume with water.

At this point in time the whole blood has been labeled with monoclonal antibody, lysed and then fixed. It now is ready for analysis with the EPICS flow cytometer equipped with the COULTER CVA, employing the principles proposed for the AMAC III, R. C. Leif et al, Clinical Chemistry 23, 1492-8 (1977); and R. A. Thomas et al, J. Histochemistry and Cytochemistry, Vol. 25, No. 77, pp 827-835 (1977). The designator COULTER "CVA" represents "cell volume accessory" and, in quite simple terms, means that the electronic cell analysis equipment utilizing the well known Coulter principle of particle detection has been utilized and integrated into a flow cytometer. Such multiparameter instrument has been demonstrated commercially. Filtration of the sample, preferably through a 37 micron mesh, is essential. The samples are stabilized on ice, if need be, for at least 5 minutes prior to sample analysis. The parameters measured were forward angle light scattering, right angle light scattering, fluorescein immunofluorescence and electronic cell volume.

Example 6

Cell Preparation: The lyse and fixative reagents are prepared at 42°C. The reagents are kept capped in a water bath throughout the procedure.

1. One hundred ul of labeling diluent consisting of 1 g of NaN_3, in 1 liter of ISOTON Plus diluent is added into a 16 x 100 tube followed by 100 ul of whole blood. The contents are then gently mixed by swirling.

2. Four micrograms of COULTER CLONE monoclonal antibody T4-FITC are added into the same tube. The tube is mixed gently, and then placed in the water bath maintained at 42°C for five minutes, shaking occasionally.

3. Then 100 ul of lyse reagent, consisting of 0.4 g saponin,

0.05 g of sorbic acid, 1.36 g of KH_2PO_4, 1.31 g of K_2HPO_4, 3.72 g of KCl, and water to make one liter, is added to the tube containing blood and antibody and continuously agitated in the water bath for 30 seconds.

4. At the end of the 30 second lysis, 1000 ul of a fixative reagent consisting of 12.6 g of NaCl, 220 g of dimethyl sulfoxide, 200 ml of Mirsky's reagent, and 600 ml of ISOTON Plus diluent, is added to the lysed sample, mixed gently, and kept in the water bath at 42°C for an additional five minutes, mixing occasionally.

At this point in time the whole blood has been labeled with monoclonal antibody, lysed and then fixed. It is ready for analysis with the EPICS flow cytometer. Filtration of the sample, preferably through a 37 micron mesh, is desirable. The samples are stabilized on ice, if need be, for at least five minutes prior to sample analysis and analyzed as stated in Example 1.

Example 7

Cell Preparation: The lyse and fixative reagents are prepared at 37°C. The reagents can be kept capped in a water bath throughout the procedure.

1. The 100 ul of a labeling diluent, consisting of 1.0 g of NaN_3, 1.36 g of KH_2PO_4, 1.31 g of K_2HPO_4 and 3.73 g of KCl, brought up to 1 liter volume with water, is added into a 16 x 100 tube followed by 100 ul of whole blood. The tube contents are then gently mixed by swirling. To this is then added 10 ul of a staining solution consisting of 0.025 g of 4,6-diamino-2-phenylindole (DAPI) in 10 ml of absolute ethanol.

2. Ten micrograms of a non-specific unlabeled mouse antibody is added to block any non-specific binding of the monoclonal antibody. The tube is mixed gently and then placed in the water bath maintained at 37°C for five minutes, shaking occasionally.

3. One microgram of COULTER CLONE monoclonal antibody T8-FITC is added into the same tube. The tube is mixed gently, and then placed in the water bath maintained at 37°C for five minutes, shaking occasionally.

4. To the tube containing the staining solution, blood and antibody, is added 100 ul of lyse reagent. This reagent consists of 4 g of saponin, 1.75 g of NaCl, 1.36 g of KH_2PO_4, 1.31 g of K_2HPO_4, 2.24 g of KCl, and water to make one liter volume. After the reagent is added, the tube is continuously agitated in the water bath for one minute.

5. At the end of the one minute lysis, 500 ul of a fixative reagent consisting of 11.7 g of NaCl, 0.43 g of calcium gluconate, 21 g of glyoxal, 220 g of dimethyl sulfoxide, 25 g of Carbowax 1450, and water to make one liter, is added to the lysed sample, mixed gently, and kept in the water bath at 37°C for an additional five minutes, mixing occasionally.

At this point in time the whole blood has been labeled with the monoclonal antibody T8-FITC and DAPI, lysed and then fixed. It is ready for preparation by centrifugal cytology. The samples are stabilized on ice, if need be, for at least 5 minutes prior to sample analysis.

6. A standard microscope slide is prepared with a poly-d-lysine solution consisting of 50 mg of poly-d-lysine having a molecular weight of approximately 700,000, by dipping the slide in the solution, and then drying on a slide dryer maintained at 60°C.

7. A pair of Leif Centrifugal Cytology buckets (U.S. Pat. 4,250,830) is assembled and the lysed blood sample is spun for five minutes at 1,500 rpm. The supernatant fluid is removed, and the sample is washed three times with the labeling diluent.

8. The supernatant fluid is removed and the cells in the labeling diluent on the slide are coverslipped. The slide is now ready for microscopic examination with a mercury arc ultraviolet excitation for DAPI, and visable excitation for FITC.

As can be seen from the preceding examples, the relative volumes of the blood sample 50 to 100 ul, the lysing reagent 100 to 1000 ul, and the fixative 500 to 1000 ul can vary. The lysing reagent 0.24 to 4 g/L and fixative concentrations 0.66 g/L to 40 g/L are inversely related to their volumes and must be scaled up as the volume of all previously added reagents is increased. The quantity of saponin

0.24 to 4.0 g/L also is inversely related to both the temperature 24°C to 60°C and the period for lysis 38 seconds to 5 minutes. The quantity of fixative also is related to whether the sample is to be stored for a prolonged period or used immediately for morphology. For the long term storage, maximum fixation is essential, but for the near immediate use, minimal fixation often is preferable.

It is understood that the illustrative embodiments set forth herein constitute examples of the principles of the present invention, but that numerous alternatives will occur to those of ordinary skill in the art, without departure from the scope of this invention.

WHAT WE CLAIM IS:

1. In a process for identifying classes and preferably also selected subclasses of leukocytes in whole blood, based on utilization of antigenic determinants on the cell surface and their reactivity with labeled antibodies, the improvement characterized by: lysing the erythrocytes with a lysing reagent containing saponin for a predetermined time and under specified temperature conditions; followed by treating the leukocytes with a cross-linking fixing reagent for a predetermined time and under specified temperature conditions; whereby the physical properties and preferably also the morphological properties of the leukocytes are maintained for enabling the leukocyte identifying.

2. The process of claim 1 characterized wherein each of said labeled antibodies consists of an antibody linked to a fluorochrome.

3. The process of claim 2 characterized wherein a combination of physical measurements is employed to isolate leukocyte classes and wherein the fluorescent distribution of each class is measured.

4. The process of claim 3 characterized wherein at least one of said physical measurements is light scattering.

5. The process of claim 3 characterized wherein at least one of said physical measurements is electronic impedance.

6. The process of claim 3 characterized wherein said physical measurements are a combination of forward angle and right angle light scattering.

7. The process of claim 3 characterized wherein said physical measurements are a combination of electronic impedance and right angle light scattering.

8. The process of claim 3 characterized wherein the leukocytes are classified by morphology, and subclassified by fluorescent intensity.

9. The process of any one of claims 1 to 8 characterized wherein said fixing reagent contains glutaraldehyde.

10. The process of claim 9 characterized wherein the concentration of said glutaraldehyde approximates 22 g per liter in the fixative solution or approximately 10 g per liter in the sample with the fixative added.

11. The process of any one of claims 1 to 8 characterized wherein said fixing reagent contains a non-fluorescing dialdehyde as the active ingredient.

12. The process of claim 11 characterized wherein said non-fluorescing dialdehyde is glyoxal.

13. The process of claim 12 characterized wherein said glyoxal is present in a concentration of 0.66 g to 40 g per liter in the fixative solution or 0.33 g to 20 g per liter in the sample with the fixative added.

14. The process of any one of claims 1 to 13 characterized by further including the labeling of specific leukocyte subclasses and then enumerating the cells in those subclasses.

15. The process of any one of claims 1 to 13 characterized wherein said leukocyte classes are determined morphologically.

16. The process of any one of claims 1 to 15 characterized wherein said lysing reagent contains saponin in a concentration of about 0.24 g per liter to about 4 g per liter; or about 0.2 g per liter to about 1.14 g per liter in the lysed sample.

17. The process of any one of claims 1 to 16 characterized wherein said lysing temperature is from about 24°C to about 42°C.

18. The process of any one of claims 1 to 17 characterized wherein said lysing time is 8 seconds to about 5 minutes.

19. A reagent system for use on a whole blood sample for identifying classes and preferably also subclasses of leukocytes which have been labeled with specific antibodies, said reagent system characterized by: a lysing reagent containing an amount of saponin sufficient for lysing the erythrocytes, but leaving the leukocytes substantially intact; and a fixing reagent containing a cross-linking agent in such amount that the physical properties and preferably also the morphological properties of the leukocytes are maintained for enabling leukocyte identifying.

20. The reagent system of claim 19 characterized wherein said fixing reagent contains glutaraldehyde.

21. The reagent system of claim 20 characterized wherein the concentration of said glutaraldehyde approximates 22 g per liter in the fixative solution, and approximately 10 g per liter in the sample with the fixative added.

22. The reagent system of claim 19 characterized wherein said fixing reagent contains a non-fluorescing dialdehyde as the active ingredient.

23. The reagent system of claim 22 characterized wherein said non-fluorescing dialdehyde is glyoxal.

24. The reagent system of claim 23 characterized wherein said glyoxal is present in a concentration of 0.66 g to 40 g per liter in the fixative solution, and 0.33 g to 20 g per liter in the sample with the fixative added.

25. The reagent system of any one of claims 19 to 24 characterized by at least one dye to stain the leukocytes.

26. The reagent system of claim 25 characterized wherein said dye is 4,6-diamino-2-pheylindole.

27. The reagent system of claim 25 characterized wherein said dye is Mithramycin.

28. The reagent system of any one of claims 19 to 27 characterized wherein said lysing reagent contains saponin in a concentration of about 0.24 g per liter to about 4 g per liter; or about 0.2 g per liter to about 1.14 g per liter in the lysed sample.

29. The reagent system of any one of claims 19 to 28 characterized wherein said lysing reagent contains sorbic acid as an additive.

30. The reagent system of any one of claims 19 to 29 characterized in which said fixing reagent also contains dimethyl sulfoxide.

31. The reagent system of claim 30 characterized wherein said dimethyl sulfoxide is present in a concentration of 0.1 to 220 g per liter in the fixative solution, and 0.1 to 125 g per liter in the sample with the fixative added.

32. The reagent system of any one of claims 19 to 29 characterized in which said fixing reagent also contains urea.

33. The reagent system of any one of claims 19 to 32 characterized by antibodies linked to a fluorochrome for labeling the leukocytes.

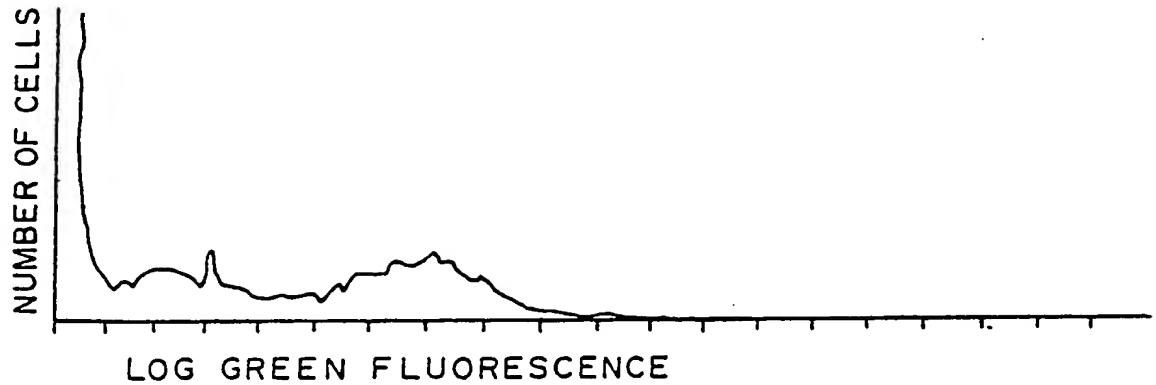

FIG. 1A.

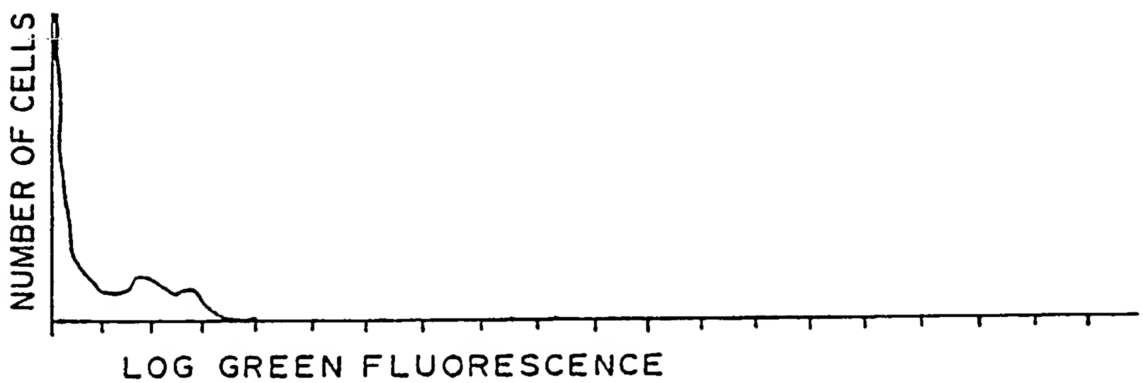

FIG. 1B.

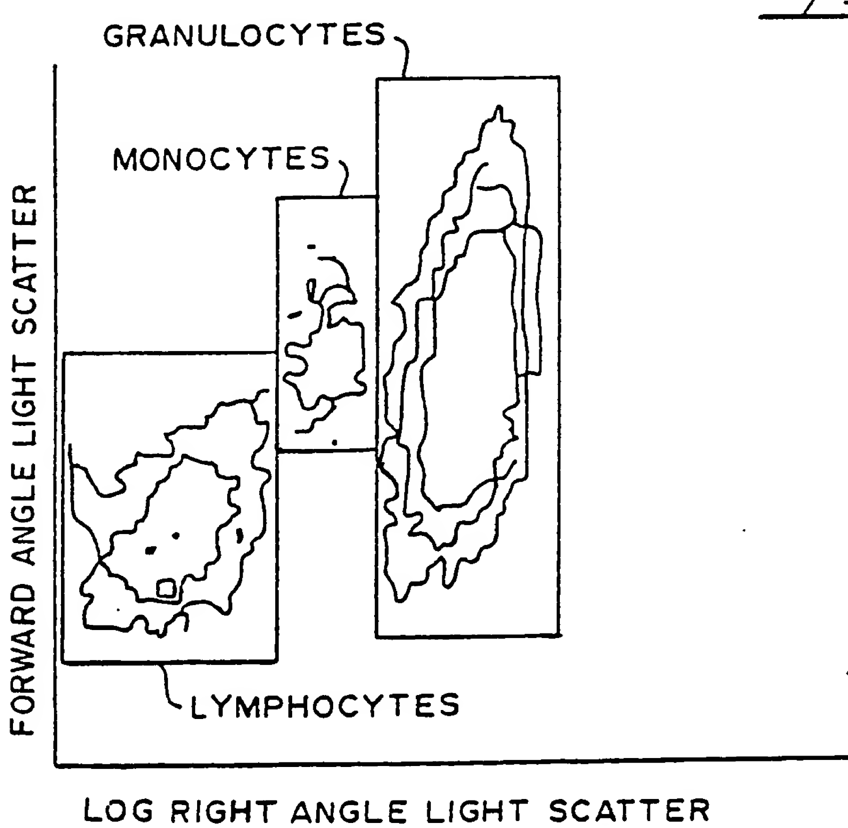

FIG. 2

INTERNATIONAL SEARCH REPORT

International Application No PCT/US85/00954

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL[4] C12Q 1/02; G01N 33/554
U.S. CL. 250/461.2; 356/39; 424/3; 435/29,34; 436/63,519

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	250/461.2; 356/39; 424/3; 435/29,34; 436/63,519

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	US,A, 3,446,751 Published 27 May 1969, Weichselbaum	1-33
A	US,A, 3,874,852 Published 01 April 1975, Hamill	
A	US,A, 3,883,247 Published 13 May 1975, Adams	
A	US,A, 3,916,205 Published 28 October 1975, Kleinerman	
A	US,A, 4,102,810 Published 25 July 1978, Armstrong	
A	US,A, 4,185,964 Published 29 January 1980, Lancaster	

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
08 August 1985	13 AUG 1985
International Searching Authority [1]	**Signature of Authorized Officer** [20]
ISA/US	Sidney Marantz

Form PCT/ISA/210 (second sheet) (October 1981)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
A	US,A, 4,286,963 Published 01 September 1981, Ledis et al	
A	US,A, 4,299,917 Published 10 November 1981, Berger et al.	
A	US,A, 4,336,029 Published 22 June 1982, Natale	
Y	US,A, 4,420,558 Published 13 December 1983, Mey	1-18
Y,P	US,A, 4,492,752 Published 08 January 1985, Hoffman et al.	1-18
Y,P	US,A, 4,499,052 Published 12 February 1985, Fulwyler	1-18
A,P	US,A, 4,529,705 Published 16 July 1985, Larsen	
Y	N, Immunology, Vol. 77, No. 8, issued August 1980, R.A. Hoffman et al. 'Simple and Rapid Measurement of Human T Lymphocytes and Their Subclasses in Peripheral Blood', pages 4914-4917	1-18
A	N, Annals New York Academy of Sciences, Vol. 420, issued 1983, D.Y.Mason et al., 'Single and Double Immuno-enzymatic Techniques for Labeling Tissue Sections with Monoclonal Antibodies', pages 127-133	
Y	N, J. Histochem. Cytochem., Vol. 25, No. 7, issued 1977, R.A.Thomas et al., 'Combined Optical and Electronic Analysis of Cells with the AMAC Transducers', pages 827-835	1-18
Y	N, Laboratroy Management, issued May 1984, J.R. Downing et al., 'Flow Cytometry: Applicants in the Clinical Laboratory', pages 29-37	1-18

International Application No. PCT/US85/00954

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
Y	N, Clin. Chem., Vol. 23, No. 8, issued 1977, R.C.Leif et al., 'Development of Instrumentation and Fluorochromes for Automated Multiparameter Analysis of Cells', pages 1492-1498	1-18
A	N, Acta Cytologya, Vol. 19, No. 4, issued 1975, G.C. Salzman et al., 'Cell Classification by Laser Light Scattering:...', pages 372-377	